The Flavor and Fragrance High Production Volume Consortia

The Terpene Consortium

Test Plan for Anethole (isomer unspecified) and *trans*-Anethole

Anethole (isomer unspecified) **CAS No. 104-46-1**

***trans*-Anethole** **CAS No. 4180-23-8**

FFHPVC Terpene Consortium Registration Number 1101125

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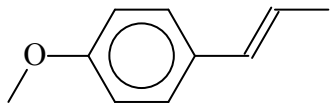
1 IDENTITY OF SUBSTANCES



Anethole (isomer unspecified)

CAS No. 104-46-1

***trans*-Anethole**

CAS No. 4180-23-8

Synonyms:
Anisole, *p*-propenyl-
4-Methoxypropenylbenzene
p-methoxypropenylbenzene
4-Propenylanisole
p-(1-propenyl)anisole
p-Propenylphenyl methyl ether

2 CATEGORY ANALYSIS

2.1 INTRODUCTION

In October of 1999, members of the U.S. flavor and fragrance industries as well as other manufacturers that produce source materials used in flavors and fragrances formed consortia of companies in order to participate in the Chemical Right-to-Know Program. Members of these consortia are committed to assuring the human and environmental safety of substances used in flavor and fragrance products. The consortia are organized as the Flavor and Fragrance High Production Volume Consortia (FFHPVC). The terpene consortium, as a member of FFHPVC, serves as an industry consortium to coordinate testing activities for terpene substances under the Chemical Right-to-Know Program. Twenty-one (21) companies are current members of the Terpene Consortium. The Terpene Consortium and its member companies are committed to assembling and reviewing available test data, developing and providing test plans for each of the sponsored chemicals, and where needed, conducting additional testing. The test plan, category analysis and robust summaries presented represent the first phase of the Consortium's commitment to the Chemical Right-to-Know Program.

2.2 BACKGROUND INFORMATION

This test plan provides data for two stereochemical forms of anethole. One is the form in which the stereochemistry is unspecified. The Chemical Abstract Registry Number (CAS No. 104-46-1) for this form encompasses both the *cis*- and *trans*- isomer. The other CAS No. (4180-23-8**)** is associated only with the *trans*- isomer. The vast majority of toxicological data is for the *trans*-anethole.

Whether obtained from natural sources (*e.g.*, anise oil or fennel oil), isolated from crude sulfate turpentine, or synthesized, anethole is mainly composed of the *trans*-isomer. *trans*-Anethole is considered by the U.S. Food and Drug Administration (FDA) [21CFR§182.60] and the Flavor and Extract Manufacturers' Association (FEMA) Expert Panel to be "generally recognized as safe" (GRAS) for its intended use as a flavoring substance [Hall and Oser, 1965]. *trans*-Anethole occurs naturally in more than 20 foods

[CIVO-TNO, 2000]. Based on recent and extensive natural occurrence data [CIVO-TNO, 2000] and annual volume of use data [Lucas *et al.*, 1999; Lawrence, 1985] intake of *trans*-anethole from consumption of traditional food approaches 100,000 kg. Anethole is formed in plants as a by-product of terpene synthesis. Although it is a minor (less than 0.5%) [Bauer and Garbe, 1985] constituent of the volatile component of turpentine, significant quantities of anethole are obtained due to the large quantities of turpentine processed.

2.3 STRUCTURAL CLASSIFICATION

Anethole is *p*-methoxypropenylbenzene or *p*-(1-propenyl)anisole. As noted above, whether produced synthetically or derived from natural sources, it occurs mainly as the *trans* isomer. Although anethole is mainly obtained from different synthetic processes [Bauer and Garbe, 1985], it can be isolated directly from crude sulfate turpentine (CST) or can be produced from the CST fraction containing estragole (*p*-methoxyallylbenzene) Isomerization of the double bond of estragole yields a mixture of *cis*- and *trans*-anethole with the *trans*- isomer predominating.

2.4 INDUSTRIAL AND BIOGENIC PRODUCTION

2.4.1 Industrial Production

Industrial sources of anethole have changed to address increasing demand. When demand was low, isolation from essential oils of fennel, anise, and star-anise played a significant role as a commercial source. Increased demand led to isolation from CST or synthetic sources such as anisole. Yields of anethole, its isomer estragole (*p*-methyoxyallylbenzene), the terpene alcohol *alpha*-terpineol, and terpene hydrocarbon caryophyllene are obtained by fractional distillation of CST. Typically, 1-2% of CST is isolated as a mixture of the four substances [Derfer and Traynor, 1992]. Further fractional yield an anethole/caryophyllene mixture accounting for approximately 0.5% of CST. Anethole can be isolated from the mixture by crystallization. The fraction of CST (approximately 1%) containing a mixture of estragole and *alpha*-terpineol can be treated with potassium hydroxide which results in the isomerization of estragole to yield an

87:13 mixture of *trans*- and *cis*-anethole. The anethole is then isolated by fractional distillation.

Route for the large-scale synthesis of anethole involves treatment of anisole (*p*-methoxybenzene) with propionic acid derivatives or propionaldehyde. In the former synthesis anisole is converted to 4-methoxypropiophenone by a Friedel-Crafts reaction with propionyl chloride [Svadkowskaya *et al*., 1970]. Reaction with propionaldehyde yields the acetal 1,1-bis(4-methoxyphenyl)propane that is subsequently hydrolyzed to anethole and anisole [Bauer and Molleken, 1974].

2.4.2 Biogenic Production

Crude sulfate turpentine (CST) is a complex mixture of C_{10} monoterpene hydrocarbons composed mainly of *alpha*-pinene (60-65%), *beta*-pinene (25-35%) and other monocyclic terpenes including a small amount of anethole (0.04 to 2%) [Millennium Chemicals, 2000]. It has been estimated that the worldwide production of turpentine is approximately 330,000 metric tons of which almost 100,000 metric tons is gum turpentine and the bulk of the remainder (230,000 tons) is sulphate turpentine [National Resources Institute, 1995]. In 1977, the annual United States production of CST and wood turpentine was reported to be 92,750 and 9,150 tons, respectively [McKibben, 1979]. The annual amount of anethole present in CST used in the United States is approximately 460 metric tons (920,000 kg).

Level-three fugacity calculations indicate that the environmental distribution of turpentine and its components is essentially entirely into the air [Mackay *et al*., 1996a, 1996b]. If it were conservatively assumed that through the various industrial processes approximately 2% is lost, the total annual worldwide emission of anethole from turpentine would be 18,400 kg. This can be compared with the biogenic emissions into the air discussed below.

As an important plant terpene by-product, anethole is a component of the earth's atmosphere [Guenther *et al*., 2000]. In determining the impact on the environment of the

industrial production and use anethole, it is also important to examine the impact as a result of emissions from biogenic sources [Guenther *et al*., 2000].

In a recent review of natural emissions of volatile compounds [Guenther *et al*., 1995] it was estimated that 70% of monoterpene flux is accounted for by pines species in North America. The total annual emission of the most common monoterpene constituent of pine (*alpha*-pinene) is approximately 4.5 million metric tons. Given that the ratio of *alpha*-pinene to anethole (0.70 to 0.02) in the volatile component of pine-derived turpentine is approximately 35, it is estimated that biogenic production is approximately 100,000 metric tons annually. Based on total annual global emission of volatile organic compounds (VOC)s [Guenther *et al*., 1995], the percentage *alpha*-pinene in the total emissions of VOCs (mean of 2.6% measured over 3 different forest types), and the pinene/anethole ratio, it can be estimated that the total annual global emissions for anethole is approximately 0.75 million metric tons (*alpha*-pinene emissions, approximately 30 million metric tons).

Based on the above estimate, it can be concluded that total annual atmospheric emission of anethole is predominantly from biogenic sources (750,000,000 kg/year of biogenic emissions *versus* 920,000 kg/year of anthropogenic emissions). The relative contribution from biogenic and industrial sources can be represented by a global emission ratio (GER = biogenic emission/industrial emission). In the case of anethole, the GER would be approximately 1,000, suggesting that biogenic emissions far exceed man-made emissions. As a result, humans are unavoidably exposed to the naturally occurring anethole.

2.5 METABOLISM OF *TRANS*-ANETHOLE

Orally administered *trans*-anethole is rapidly absorbed, undergoes nearly complete metabolism in the liver producing metabolites that are conjugated and then excreted primarily in the urine. Some elimination as CO_2 in expired air also occurs [Fritsch *et al*., 1975; Le Bourhis, 1968, 1970, 1973a; Solheim and Scheline, 1973, 1976]. In the rat,

major urinary and intermediary metabolites enter hepatic circulation *via* bile [Solheim and Scheline, 1976].

The pharmacokinetic and metabolic pathways of *trans*-anethole have been well studied in humans, mice and rats and extensively reviewed [Newberne *et al*., 1999]. Three principal pathways of detoxication are followed (*omega*-oxidation, *O*-demethylation, and epoxidation; see Figure 1) and are dependent on dose, animal species, sex, and exposure duration.

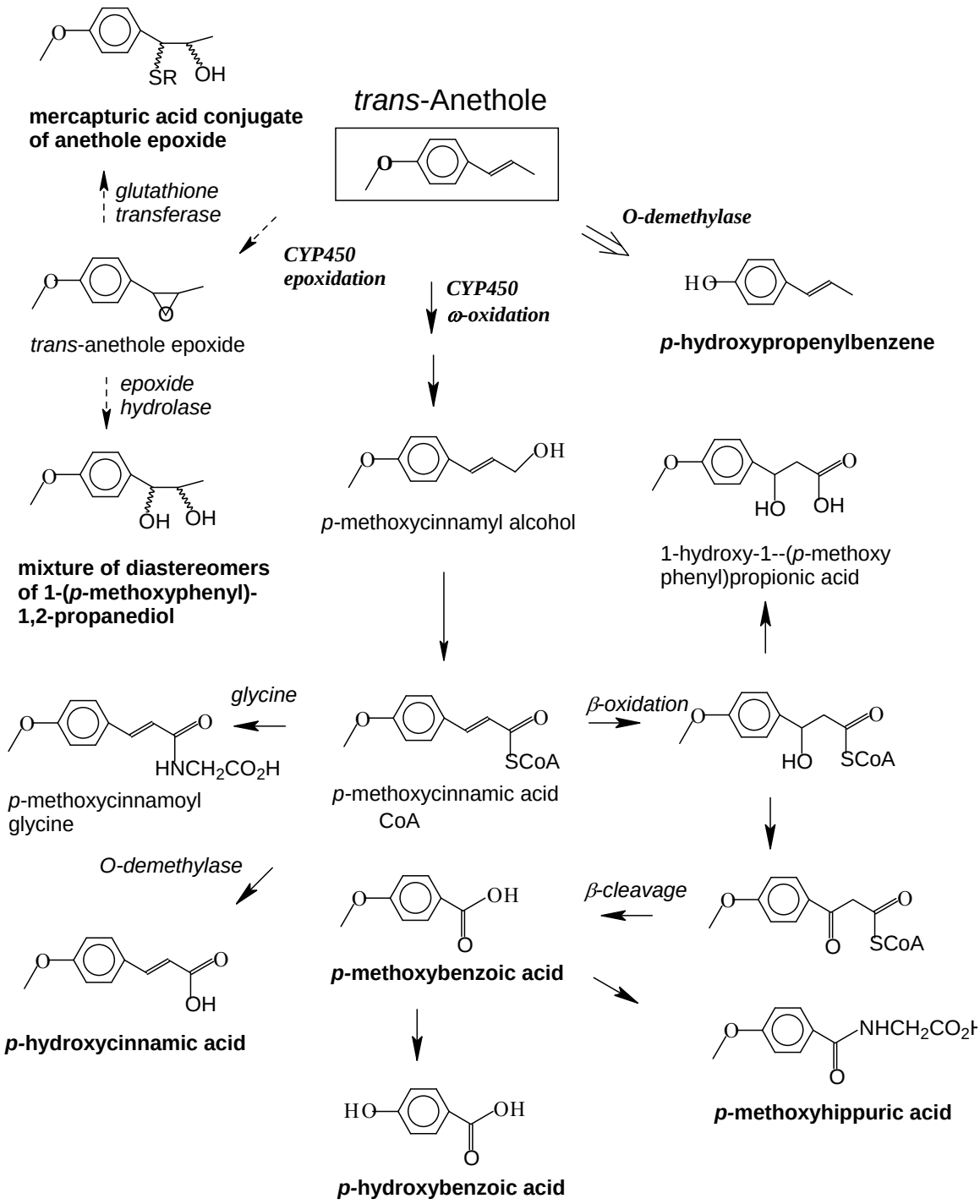
In humans, a single dose of *trans*-anethole was excreted primarily in the urine (60%) following *omega*-oxidation yielding *p*-methoxyhippuric acid (52-56%) and *p*-methoxybenzoic acid within 24 hours (3.5-5%) [Le Bourhis, 1973a; Sangster *et al*., 1987]. Similar results were reported when 3 separate doses of *trans*-anethole were administered. In addition, no dose-dependency on the rate or route of excretion was noted [Caldwell and Sutton, 1988]. About 21% of a 0.05 mg dose of *trans*-anethole was metabolized by *O*-demethylation and expired as CO_2, while approximately 3% of the dose was metabolized to an epoxide [Sangster *et al*., 1987]. The findings of these studies indicated that, when administered to humans at 0.05 to 12 mg/kg bw, *trans*-anethole undergoes metabolic detoxication by *omega*-oxidation and *O*-demethylation.

In mice, an intraperitoneal injection of 50 mg *trans*-anethole/kg bw was excreted in the urine (37%), expired air as CO_2 (47%), and in the feces (less than 2%) within 24 hours [Sangster *et al*., 1984a]. *O*-Demethylation was the critical pathway yielding CO_2 and various urinary metabolites. *omega*-Oxidation accounted for approximately 17.8% of the dose as *p*-methoxyhippuric acid and epoxidation resulted in approximately 6.2% of the dose. When the effect of dose was examined over the range of 0.05 to 1,500 mg/kg bw, a significant shift in metabolism from *O*-demethylation (71.8% at low dose to 34.6% at high dose) to *omega*-oxidation (10.4% at low dose to 43.8% at high dose) and to a lesser extent, epoxidation (1.6% at low dose to 8.8% at high dose) was observed [Sangster *et al*., 1984b]. Also, elimination was slower at higher dose levels (24 hours at low dose and 72 hours at high dose). The effect of dose, sex and pre-feeding also was examined in mice pre-fed *trans*-anethole 62-426 mg/kg bw for 21 days, followed by a single oral

gavage dose (equivalent to the pre-feeding level determined from week 3 of the study) [Bounds, 1994; Bounds and Caldwell, 1992, 1996]. The majority of the dose (75-90%) was excreted in the urine of control and treated mice within 24 hours and no effect of dose was noted. At the high dose, pre-fed males tended to excrete more *omega*-oxidation metabolites than females. In addition, glycine conjugation of *p*-methoxycinnamic acid decreased with increasing dose and pre-feeding, particularly in females. Accompanying this decrease was a dose-related increase in the formation of glutathione conjugates. Only a slight, but significant, increase in epoxide conjugates was observed in high-dose mice, notably females. The findings of these studies indicate that the major detoxication pathways in mice are *omega*-oxidation and *O*-demethylation.

In rats administered a single oral dose of *trans*-anethole 50 mg/kg bw, *O*-demethylation (41.8%), *omega*-oxidation (12.8%) and epoxidation (14.3%) metabolites were excreted in the urine [Sangster *et al*., 1984a]. Female rats tended to eliminate an intraperitoneal dose of *trans*-anethole 250 mg/kg bw slower than male rats, since after 24 hours, 56% of the dose was recovered in the urine of female rats compared to 71% recovered in male rats [Caldwell, 1991; Caldwell *et al*., 1991]. When the effect of dose was examined over the range of 0.05 to 1,500 mg/kg bw, a significant shift in metabolism from *O*-demethylation (56% at low dose to 32% at high dose) to side-chain *omega*-oxidation (2.6% at low dose to 17.5% at high dose) and to epoxidation (3% at low dose to 18% at high dose) was observed [Sangster *et al*., 1984b]. The effect of dose, sex and pre-feeding also was examined in rats pre-fed *trans*-anethole 100-1,000 mg/kg body for 3 weeks, followed by a single oral gavage dose (equivalent to the pre-feeding level determined from week 3 of the study) [Bounds, 1994; Bounds and Caldwell, 1992, 1996]. In non-pre-fed rats (controls), the rate of elimination of urinary metabolites decreased with increasing dose (80% at low dose *versus* 50% at high dose); whereas, in pre-fed rats, the rate remained essentially constant suggesting that pre-feeding increases the capacity of rats to metabolize and eliminate *trans*-anethole. Overall, detoxication of *trans*-anethole in the rat is dose-dependent and tends to shift from *O*-demethylation to *omega*-oxidation and, more importantly, epoxidation at higher doses.

Figure 1. Metabolism of *trans*-anethole in humans, rats and mice



2.6 SUMMARY FOR CATEGORY ANALYSIS

trans-Anethole, a natural component of the diet, is readily absorbed, metabolized and rapidly excreted *via* the urine as *O*-demethylation and *omega*-oxidation metabolites and, to some extent, epoxidation metabolites. The physiochemical properties and low toxic potential of *trans*-anethole are consistent with its known reactivity and metabolic fate.

3 TEST PLAN

3.1 CHEMICAL AND PHYSICAL PROPERTIES

3.1.1 Melting Point

The melting point of anethole (isomer unspecified) has been reported to be 21.3 °C [CRC, 1995] and for *trans*-anethole 21.4 °C [Merck Index, 1997]. The calculated melting point for *trans*-anethole according to the MPBPWIN program was –0.69 °C [MPBPVP EPI Suite, 2000]. Based on these reported values the melting point of anethole (isomer unspecified) or *trans*-anethole is 21.3-21.4 °C.

3.1.2 Boiling Point

The boiling point of anethole (isomer unspecified) has been reported to be 234 °C [CRC, 1995] and for *trans*-anethole 236 °C [FMA]. The calculated boiling point for *trans*-anethole according to the MPBPWIN program was 217.31 °C [MPBPVP EPI Suite, 2000]. Based on the consistency of these values, the boiling point of anethole (isomer unspecified) or *trans*-anethole is 234-236 °C.

3.1.3 Vapor Pressure

The vapor pressure of anethole (isomer unspecified) has been reported to be 0.041 (5.45 Pa) at 21 °C [Daubert and Danner, 1989]. The calculated vapor pressure of *trans*-anethole has been reported to be 0.05 mm Hg (6.67 Pa) at 20 °C [FMA] and according to the MPBPWIN program was 0.0634 mm Hg (8.45 Pa) at 25 °C [MPBPVP EPI Suite, 2000]. Based on these data the vapor pressure of anethole (isomer unspecified) or *trans*-anethole is approximately 0.05 mm Hg (6.67 Pa) at 20 °C.

3.1.4 n-Octanol/Water Partition Coefficients

Log KOW of *trans*-anethole was calculated resulting in values of 3.39 [KOWWIN EPI Suite, 2000] and 3.11 [Interactive Analysis LogP and LogW Predictor]. The close

agreement between calculated values indicated that the log KOW for *trans*-anethole is 3.11-3.39.

3.1.5 Water Solubility

The water solubility of anethole (isomer unspecified) was reported to be 111 mg/L at 25 °C [WSKOW EPI Suite, 2000a]. Water solubility was also calculated for *trans*-anethole resulting in a value of 285.4 mg/L [Interactive Analysis LogP and LogW Predictor] and 139.8 mg/L at 25 °C [WSKOW EPI Suite, 2000b]. Based on these data the water solubility of anethole (isomer unspecified) or *trans*-anethole is approximately 111 mg/L at 25 °C.

3.1.6 New Testing Required

None.

3.2 ENVIRONMENTAL FATE AND PATHWAYS

3.2.1 Photodegradation

The calculated half-life value for *trans*-anethole has been reported to be 2.015 hours [AOPWIN EPI Suite, 2000]. The fact that anethole contains a reactive allylic hydrogen capable of ready reaction with hydroxyl and peroxy radicals supports the calculated short half-life.

3.2.2 Stability In Water

No hydrolysis is possible for anethole. Anethole (isomer unspecified) or *trans*-anethole is expected to be stable in aqueous solution.

3.2.3 Biodegradation

Anethole (isomer unspecified) demonstrated ready and ultimate biodegradability using a CO2 production test based on OECD Guideline 301B. Biodegradation was 91.0% (90.7-91.2%). [Quest International, 1994].

3.2.4 Fugacity

Transport and distribution in the environment were modeled using Level III Fugacity-based Environmental Equilibrium Partitioning Model [Mackay *et al*., 1991a, 1996b] through the EPA EPI suite 2000 program. The input parameters used were molecular weight, vapor pressure (0.41 mm Hg) measured melting point (21.35°C) and boiling point (234°C).

The model predicts that *trans*-anethole is distributed mainly to the soil (69.1%), but also is distributed to water (29.8%) and, to some extent, air (0.53%) and sediment (0.60%).

The significance of these calculations must be evaluated in the context that *trans*-anethole is a product of plant biosynthesis and is, therefore, ubiquitous in the environment. The model does not account for the influence of biogenic production or

chemical reactivity on partitioning in the environment nor does it take into account any biodegradation.

3.2.5 New Testing Required

None.

3.3 ECOTOXICITY

3.3.1 Acute Toxicity to Fish

Suitable measured and calculated fish LC50s were available for *trans*-anethole. In fathead minnows, the 96-hour LC50 was determined to be 7.690 mg/L with an EC50 of 4.810 mg/L [Broderius *et al*., 1990]. The calculated 96-hour LC50 was reported to be 5.423 mg/L (neutral organics) and 2.433 mg/L (SW) and the 14-day LC50 was reported to be 12.251 mg/L [ECOSAR EPI Suite, 2000].

Given the current database of information, it will not be necessary to perform additional acute fish toxicity tests.

3.3.2 Acute Toxicity to Aquatic Invertebrates

Measured and calculated aquatic invertebrate LC50s were available for *trans*-anethole. In *Daphnia magna,* the 48-hour LC50 was determined to be 6.82 mg/L with a 48-hour EC50 of 4.25 mg/L [Broderius *et al*., 1990]. In addition, calculated values were reported for 48-hour LC50 of 6.397 mg/L and a 16-day EC50 of 0.603 mg/L [ECOSAR EPI Suite, 2000]. A calculated 96-hour LC50 of 0.580 mg/L was reported for mysid shrimp [ECOSAR EPI Suite, 2000].

Given the current database of information, it will not be necessary to perform additional acute aquatic invertebrate toxicity tests.

3.3.3 Acute Toxicity to Aquatic Plants

Measured and calculated values for aquatic plants are available for *trans*-anethole. In green algae, a 96-hour IC50 of 9.571 mg/L was determined [Broderius *et al*., 1990]. A calculated 96-hour EC50 of 4.332 mg/L was reported for green algae [ECOSAR EPI Suite, 2000].

Given the current database of information, it will not be necessary to perform additional acute aquatic plant toxicity tests.

3.3.4 New Testing Required

None.

3.4 HUMAN HEALTH TOXICITY

3.4.1 Acute Toxicity

In rats, mice and guinea pigs, anethole (isomer unspecified and *trans*-) showed low acute toxicity with oral LD50s of 2,090-3,200 mg/kg bw for rats, 3,050-5,000 mg/kg bw for mice, and 2,160 mg/kg bw for guinea pigs, and intraperitoneal LD50s of 703-900 mg/kg bw for rats and 650-1,410 mg/kg bw for mice [Caujolle and Meynier, 1958; Jenner *et al*., 1964; Boisser *et al*., 1967; Borriston Laboratories Inc., 1984].

Mild liver lesions were reported in male and female rats gavaged with anethole 695 mg/kg bw/day for 4 days [Taylor *et al*., 1964].

Given the studies available, additional acute toxicity tests in mammals are not recommended.

3.4.2 *In vitro* and *In vivo* Genotoxicity

3.4.2.1 In vitro

Anethole (*trans*- and isomer unspecified) was tested extensively in various *Salmonella typhimurium* strains (TA98, TA100, TA1535, TA1537, or TA1538) at concentrations of up to 600 micrograms/plate in the presence or absence of metabolic activation. *Salmonella typhimurium* strains TA98, TA1535, TA1537, and TA1538 consistently produced negative results [Hsia *et al*., 1979; Nestmann *et al*., 1980; Sekizawa and Shibamoto, 1982; To *et al*., 1982; Mortelmans *et al*., 1986; Heck *et al*., 1989]. Mixed results were reported with *Salmonella typhimurium* strain TA100 showing positive results in the presence of S9 [Sekizawa and Shibamoto, 1982] or S13 [Swanson *et al*., 1979] and negative results in the presence or absence of a metabolic activation system [Hsia *et al*., 1979; Nestmann *et al*., 1980; To *et al*., 1982; Mortelmans *et al*., 1986; Heck *et al*., 1989; Gorelick, 1995].

In a supplementary study, the cofactor 3'-phosphadenosine-5'-phophosulfate (PAPS) was added and significantly increased the mutagenic activity of *trans*-anethole in *Salmonella typhimurium* strain TA1535 [To *et al*., 1982]; however, this positive finding was not repeated in a more recent study by Gorelick [1995].

Anethole (isomer unspecified) did not induce revertants in the *Escherichia coli* WP2 *uvrA* reversion assay when tested at concentrations of up to 600 micrograms/plate and produced negative results in the *Bacillus subtilis* DNA repair test at a concentration of 10 mg/disk [Sekizawa and Shibamoto, 1982].

trans-Anethole did not produce a response when tested in *Saccharomyces cerevisiae* strains D7 or XV185-14C in the absence of metabolic activation [Nestmann and Lee, 1983].

In rat hepatocytes, anethole (isomer unspecified) did not induce unscheduled DNA synthesis (UDS) when tested at concentrations up to 0.01 M [Heck *et al*., 1989; Marshall *et al*., 1989; Howes *et al*., 1990; Caldwell *et al*., 1992; Marshall and Caldwell, 1996]. In one study with rat hepatocytes, *trans*-anethole showed a very slight response in the UDS assay at a concentration of 0.001 M but showed no response at lower concentrations and was cytotoxic at 0.01 M [Műller *et al*., 1994].

No significant increase in mutant frequency was reported when up to 72 micrograms/ml of *trans*-anethole was tested in L5178Y/TK+/- mouse lymphoma cells in the absence of S9; however, in the presence of S9, *trans*-anethole produced a concentration-dependent increase in mutant frequency paralleled by a decrease in total growth [Gorelick, 1995]. Similar results were reported by Heck *et al*. [1989].

trans-Anethole produced no significant increase in chromosomal aberrations in Chinese hamster ovary (CHO) cells with or without metabolic activation at concentrations of up to 0.2 microliters/ml [Gorelick, 1995].

3.4.2.2 In vivo

Anethole (*trans*- and isomer unspecified) was tested *in vivo* in rats and mice for its potential to affect DNA. Unscheduled DNA synthesis (UDS) was not reported in the hepatocytes of rats gavaged with *trans*-anethole up to 500 mg/kg bw [Marshall and Caldwell, 1996]. Intraperitoneal injections of anethole up to 10 mg/mouse produced only low levels of DNA adducts in adult and newborn mice [Phillips *et al*., 1984; Randerath *et al*., 1984].

When tested in the micronucleus assay, anethole (isomer unspecified) at doses of up to 1,000 mg/kg bw/day over 7 days did not increase the frequency of micronuclei or affect the ratio of polychromatic erythrocytes to normochromatic erythrocytes in the femoral cells of mice [Al-Harbi *et al*., 1995]. It was concluded that anethole (isomer unspecified) was non-clastogenic.

3.4.2.3 Conclusions

The genotoxicity database on anethole (*trans*- and isomer unspecified) shows no mutagenic potential in the Ames assay. In cytogenetic assays, there is no evidence of a genotoxic potential *in vitro*. In whole animals, the genotoxicity results for anethole showed no micronuclei induction in mice, no UDS response in rats, and limited potential to form DNA adducts in mice. Based on these results no additional genotoxicity tests are recommended.

3.4.3 Repeat Dose Toxicity

Numerous short- and long-term rodent studies have been conducted to evaluate the safety of *trans*-anethole because of its important use as a flavoring substance. Most of the studies conducted were comprehensive, but some also focused on potential hepatic effects due to the results of metabolism studies that indicated that anethole might be a hepatotoxin in rats.

3.4.3.1 *Subacute Studies*

In a preliminary dose range-finding study, groups of 5 rats/sex were fed *trans*-anethole up to 1,200 mg/kg bw/day in the diet for a period of 28 days [Minnema, 1997a]. No notable treatment-related findings were reported at doses up to 300 mg/kg bw/day, although decreased feed consumption was noted in the early part of the study. At the higher doses, in addition to decreased body weight and feed consumption (due to poor palatability of diet), some hepatic effects, as indicated by serum biochemistry results and microscopic examination, were reported.

In a preliminary dose range-finding study, groups of 5 mice/sex were fed *trans*-anethole up to 500 mg/kg bw/day in the diet for a period of 28 days [Minnema, 1997b]. Decreased feed consumption seen at doses of 120 mg/kg bw/day and higher were associated with the poor palatability of the diet. At doses of 240 mg/kg bw/day and above, some mice stopped eating, morality increased (greater than 40%), and decreased body weights were reported, particularly in male mice. No treatment-related histomorphological changes were observed in the liver at any dose. The results from this study appear to be related to the poor palatability of *trans*-anethole in the diet and compromised food intake.

3.4.3.1.1 Special Studies on Immunosuppression

Three studies in mice were conducted to assess the immunosuppressive potential of anethole in mice.

Groups of 20 female mice were gavaged with *trans*-anethole up to 750 mg/kg bw/day for 5 days [IIT Research Institute, 1995a]. On the third day of treatment, mice were also injected intravenously with *Listeria monocytogenes*. No statistically significant differences in mortality or time to death in treated mice compared to controls. *trans*-Anethole did not affect the ability of mice to withstand a *Listeria monocytogenes* challenge.

Groups of 10 female mice were gavaged with *trans*-anethole up to 750 mg/kg bw/day for 5 days [IIT Research Institute, 1995b]. Four days prior to *trans*-anethole treatment, mice were injected intraperitoneally with sheep red blood cells (SRBC) and again after the 5

days of *trans*-anethole treatment. Four days after last intraperitoneal injection, mice were killed and spleens were removed. Plaque-forming cells (PFC) were determined from diluted spleen cells. Absolute thymus weight was significantly decreased in high-dose mice. No other effects were noted. The results of this study indicated that *trans*-anethole did not affect the ability of mice to generate antibody plaque-forming cells following immunization with sheep red blood cells.

In another similar study, groups of 8 male mice were gavaged with anethole 875 mg/kg bw/day for 11 days [Borriston Laboratories, Inc., 1982]. On the third day of treatment, mice were intraperitoneally injected with 0.3 ml 25% SRBC. On day 12 of the study, the mice were killed and the spleen, thymus and adrenals were removed and weighed. Serum was also isolated and tested for hemagglutinating activity to SRBC. There were no differences in spleen, thymus and adrenal organ weights or in the agglutination scores and calculated antibody index when compared with control values. Anethole was not immunosuppressive in this assay.

3.4.3.1.2 Special Studies on Enzyme Induction

The findings from metabolism and repeat-dose studies indicated that *trans*-anethole might have hepatotoxic effects in the rat, which resulted in examination of its potential to be an enzyme inducer in the liver.

Groups of 7 female rats were gavaged with anethole up to 300 mg/kg bw/day in corn oil for 4 days [Wenk, 1994]. On the fifth day, body weights were taken, rats were killed and livers were removed and homogenized. The homogenate was centrifuged and the supernatant (S9) was used to determine P450 and P448 activity. Enzyme activity was determined using 3 assays: *p*-nitroanisole *O*-demethylation (PNAS), 7-ethoxycoumarin *O*-deethylation (7EC), and ethoxyresorufin *O*-deethylation (EROD). There were no statistically significant differences in body weight or absolute and relative liver weight in treated rats compared to controls. The activities were statistically significant for PNAS and EROD at 300 mg/kg bw/day. In this assay, anethole induced cytochrome P450 and P448 hepatic activity in rats.

Groups of 24 female rats were injected intraperitoneally with *trans*-anethole 300 mg/kg bw/day for 7 days [Reed and Caldwell, 1992a] (No robust summaries were prepared for related biochemical studies in Reed, 1994). Twenty-four hours following last injection, rats were killed, livers were removed and weighed, and hepatic microsomes were prepared. Cytochrome P450 activity was determined using 7-ethoxycoumarin *O*-deethylase. There was a significant (p less than or equal to 0.05) increase in relative liver weights, microsomal protein (18% increase) and in microsomal cytochrome P450 (45% increase) in anethole-treated rats compared to vehicle controls. The authors concluded that *trans*-anethole has a modest enzyme-inducing effect on rat liver.

To further study this effect, groups of 8 rats/sex were fed up to 1.0% *trans*-anethole in the diet for 21 days [Reed and Caldwell, 1992a] (No robust summaries were prepared for related biochemical studies in Reed and Caldwell, 1992b and Reed, 1994). Additional groups of 5 rats/sex were treated similarly, but were allowed to resume the untreated basal diet for 14 days after the 21-day treatment period. Rats were killed, livers were removed and weighed, and hepatic microsomes were prepared. Hepatic microsomal protein and cytochrome P450 levels were determined using cytochrome C reductase, ethoxycoumarin *O*-deethylase, ethoxy and pentoxyresurufin *O*-dealkylase activities. For the rats killed on day 22, significant (p less than or equal to 0.05) increases in relative liver weight were reported in females and males at the two highest doses. Mean protein levels and cytochrome P450 content were significant (p less than or equal to 0.05) at all doses in treated females and at the two highest doses in males. For the rats undergoing a 14-day recovery period, there were no significant differences to controls with the exception of one (considered to be anomalous and related to the lack of sensitivity of the assay) finding of increased cytochrome P450 activity in females fed 0.25% *trans*-anethole. The authors concluded that *trans*-anethole has a modest enzyme-inducing effect on rat liver and noted that female rats tend to be more sensitive. In addition, these effects were reversible when anethole exposure was terminated.

In a supplementary study to determine whether biochemical changes are associated with cell proliferation, groups of 5 rats/sex also were fed up to 1.0% *trans*-anethole in the diet for 21 days [Reed and Caldwell, 1992a] (No robust summaries were prepared for related

biochemical studies in Reed and Caldwell, 1992b and Reed, 1994). For the last 3 days of *trans*-anethole exposure, 3 rats/sex/group were given 20 micrograms 5-bromo-2'-deoxyuridine (BrdU) subcutaneous *via* osmotic mini-pumps. Rats were killed and livers were removed. Liver sections were taken and treated with a murine anti-BrdU mAb plus a peroxidase-conjugated second antibody. Preliminary data indicate that liver sections from female rats fed the 0.5% *trans*-anethole diet contain higher numbers of labeled cells than control or rats fed 0.25% *trans*-anethole diet. Conversely, high-dose female rats appear to have fewer labeled cells than the other dose groups. No significant effects reported for males.

Enzyme induction was also studied in mice. Groups of 24 mice/sex were fed 0, 0.1, 0.25, 0.5 or 1.0% *trans*-anethole in the diet for 22 days [Reed and Caldwell, 1993]. Mice in the 1.0% group were terminated prematurely due to severe weight loss. The remaining mice were killed, livers were removed and weighed, and hepatic microsomes were prepared. The diet was unpalatable to the mice resulting in decreased body weight at 0.25 and 0.5%. Relative liver weights were significantly (p less than or equal to 0.005) increased at the two lowest doses, but not at 0.5%. Microsomal protein was significantly (p less than or equal to 0.005 and p less than or equal to 0.05) increased in males given 0.25 and 0.5. Cytochrome P450 was significantly (p less than or equal to 0.005 and p less than or equal to 0.05) increased in males of the 0.5% group and in females of the 0.25 and 0.5% groups. Since caloric restriction is known to induce hepatic cytochrome P450, a similar study was conducted using 0.5% *trans*-anethole but restricted the dietary intake of control mice to that consumed by the treated mice. The comparison of microsomal cytochrome P450 content in these mice still showed a significant (p less than or equal to 0.05) increase over controls. The authors concluded that *trans*-anethole has a modest enzyme-inducing effect on mouse liver.

3.4.3.2 Subchronic Studies

The only effect reported in rats fed 10,000 ppm anethole in the diet for 15 weeks were slight hydropic microscopic changes of hepatocytes in male rats [Hagan *et al*., 1967].

In a series of studies with newborn mice, the authors examined the potential of *trans*-anethole to produce hepatomas when administered through various routes of exposure at an early age. *trans*-Anethole consistently showed no hepatocarcinogenic activity when administered to mice prior to weaning, as described below.

Four-day-old mice were gavaged with *trans*-anethole 0, 2.5 or 5 micromol/kg bw twice weekly for a total of 10 times and were killed between 11 and 14 months of age [Miller *et al*., 1983]. No statistically significant change in the percent of hepatoma-bearing mice, average number or hepatomas/mouse, or number of mice with lung adenomas compared to control values was reported.

Male mice were intraperitoneally injected at 1, 8, 15 and 22 days of age with *trans*-anethole resulting in a total dose of 4.75 micromol [Miller *et al*., 1983]. Some mice were examined by laparotomy at 13 months and those surviving were killed at 18 months of age and examined for induction of hepatomas. No statistically significant change in the percent of hepatoma-bearing mice, or average number of hepatomas/mouse compared to control values was reported.

Another group of male mice were intraperitoneally injected at 1, 8, 15 and 22 days of age with *trans*-anethole resulting in a total dose of 9.45 micromol [Miller *et al*., 1983]. Mice were weaned at 22 days of age, killed at 12 months of age and examined for induction of hepatomas. No statistically significant change in the percent of hepatoma-bearing mice, average number of hepatomas/mouse, or number of mice with lung adenomas compared to control values was reported.

In a separate experiment, female mice were given *trans*-anethole 1 micromol/kg bw by intraperitoneal injection, twice/week for a total of 24 injections and after 8 months were examined for the development of lung adenomas [Miller *et al*., 1983]. No statistically significant change in the percent of mice with lung adenomas or the average number of adenomas/mouse compared to control values was reported. *trans*-Anethole showed no pulmonary carcinogenic activity when administered to mice over 12 weeks.

Comprehensive, GLP-compliant 90-day studies have been conducted in rats and mice [Minnema, 1997c; Minnema, 1997d]. In rats fed *trans*-anethole up to 900 mg/kg bw/day *via* the diet, body weights, feed consumption, and feed efficiency were decreased in males fed 300 mg/kg bw/day or more and in females fed 600 mg/kg bw/day or more. The authors attributed these effects to the poor palatability of the treated diet. Relative liver weights showed statistically significant increases in rats fed 300 mg/kg bw/day or more. Additional effects reported included centrilobular to diffuse hepatocellular hypertrophy in (males at greater than or equal to 300 mg/kg bw/day; females at greater than or equal to 600 mg/kg bw/day), minimal to slight single cell hepatocellular necrosis associated with perivascular inflammatory infiltrate (males at greater than or equal to 600 mg/kg bw/day; females at greater than or equal to 900 mg/kg bw/day), increased blood *gamma*-glutamyltranferase (males at greater than or equal to 900 mg/kg bw/day; females at greater than or equal to 600 mg/kg bw/day), and increased alanine and aspartate aminotransferase (females at greater than or equal to 900 mg/kg bw/day). These latter effects were considered to be adaptive physiological responses associated with the enzyme induction properties of *trans*-anethole (see section 3.4.3.1.2), rather than adverse effects. Based on the observed necrosis in males and increased levels of *gamma*-glutamyltranferase in females, the no observable adverse effect level (NOAEL) was reported to be 300 mg/kg bw/day.

Similarly, mice were fed up to 240 mg *trans*-anethole/kg bw/day *via* the diet [Minnema, 1997c]. Severe loss of body weight and dehydration were reported mainly at doses of greater than or equal to 120 mg/kg bw/day and were attributed to inanition syndrome (starved mouse syndrome) resulting from the poor palatability of the diet and reduced food intake. Other effects reported included liver glycogen depletion (males at greater than or equal to 30 mg/kg bw/day; females at greater than or equal to 60 mg/kg bw/day), decreased mean cell volume (males at greater than or equal to 120 mg/kg bw/day), decreased mean cell hemoglobin (males at greater than or equal to 120 mg/kg bw/day), reduced cellularity of the spleen (males at 240 mg/kg bw/day), delayed kidney development (males at 240 mg/kg bw/day), increased absolute and relative liver weights (males at greater than or equal to 30 mg/kg bw/day), increased relative thyroid weight

(males at greater than or equal to 30 mg/kg bw/day), decreased absolute spleen weight (males at greater than or equal to 60 mg/kg bw/day), decreased relative (to brain) spleen weight (males at greater than or equal to 60 mg/kg bw/day), decreased absolute and relative (to brain) kidney weights (males at greater than or equal to 120 mg/kg bw/day), increased absolute and relative adrenal weights (males at greater than or equal to 60 mg/kg bw/day), decreased absolute heart and adrenal weights (females at 240 mg/kg bw/day), increased incidence of centrilobular hepatocellular hypertrophy (males at greater than or equal to 60 mg/kg bw/day), and increased serum alkaline phosphatase (males at greater than or equal to 120 mg/kg bw/day). As in the rat, the enlarged livers, increased liver weight, and increased incidence of centrilobular hepatocellular hypertrophy were considered to be adaptive physiological responses to the enzyme inducing effect of *trans*-anethole. Increased serum alkaline phosphatase was considered also to be an adaptive response, or related to the reduced feed intake. The decreased values for mean cell volume and mean cell hemoglobin were not accompanied by significant differences in mean erythrocyte count, hemoglobin or hematocrit. In addition, the decrease was of low magnitude and therefore, the changes were considered incidental. The authors determined the NOAEL to be 240 mg/kg bw/day based on the lack of treatment-related adverse effects. An independent histopathological evaluation on the livers of the rats and mice from the 90-day studies concurred with these conclusions [Newberne, 1997] (no robust summary prepared).

3.4.3.3 Chronic Studies

No effects were reported in rats fed 2,500 ppm anethole for one year [Hagan *et al*., 1967].

Groups of rats were fed up to 1.0% *trans*-anethole in the diet (approximately up to 400 and 550 mg/kg bw/day for males and females, respectively) for up to 177 weeks [Truhaut *et al*., 1989]. An additional group of 26 rats/sex was fed 1% *trans*-anethole until week 54 and then received basal diet only until the end of the study. Between weeks 42-45, most rats showed signs of sialodacryoadenitis resulting in transient retardation of body weight gain. All treated groups showed lower body weight gains. The reversal group showed no difference in body weight gain compared to controls by the end of the study. Mortality

was increased in high-dose females and reduced adiposity was reported in high-dose rats, particularly males. No effect on hematological parameters was reported. Notable non-neoplastic effects on the liver included sinusoidal dilatation (at 0.5 and 1%); nodular hyperplasia (at 0.5 and 1% in males and 1% in females); and hepatocytic hypertrophy (at 0.5 and 1% in females). The only statistically significant finding in neoplastic lesions was an increase in the incidence of liver tumors in 1% females, but the authors noted that the increased incidence of hepatocellular carcinomas reported in high-dose females were "late onset", had no effect on longevity and was still within the range of historical controls. The reduced adiposity was considered to be an indirect effect of the poor palatability of the treated diet and decreased feed consumption. The authors determined a NOAEL of 100 mg/kg bw/day for males based on nodular hyperplasia and 120 mg/kg bw/day in females based on sinusoidal dilatation and hepatocellular hypertrophy and concluded that there was insufficient evidence to conclude that *trans*-anethole is a human carcinogenic risk. An independent group of pathologists re-evaluated the pathology data and concurred with the conclusion [Newberne, 1989] (no robust summary prepared).

Female mice were fed 0.46% trans-anethole in the diet either with or without concurrent exposure to 0.05% phenobarbital in drinking water [Miller *et al*., 1983]. Anethole exposure was stopped at 12 months. Mice were killed after 18 months and examined for induction of hepatomas. There was no statistically significant change in the average number of hepatomas/mouse compared to control values regardless of exposure to phenobarbital.

Given the numerous and comprehensive repeat-dose studies available on anethole, further testing with subacute, subchronic, and chronic protocols is not recommended.

3.4.4 Reproductive Toxicity

Anethole was tested for its potential reproductive toxicity in a comprehensive 4-generation rat study [Le Bourhis, 1973b]. In this study, groups of male and female rats (F_0) were fed 0 or 1% anethole in the diet (approximately 600-1,500 mg/kg bw/day) prior to mating, during the 15-day mating period, and during gestation and lactation. Offspring

(F_1) were used for propagating the next generation and were raised on the same dietary treatment as their parents. A similar procedure was followed to obtain the 3rd and 4th generations (F_2 and F_3). The only notable effect was reduced body weight gain and body weights coinciding with reduced feed intake in rats fed 1% anethole. There was no effect on reproductive performance over 4 generations. The reduced palatability of the diet was considered to be responsible for the lower body weight gain and body weights of the rats receiving anethole.

To ascertain the effect of palatability on the effects reported in the 4-generation study, a cross-fostering experiment was conducted using groups of control and treated F_1 females (from the 4-generation study and receiving 1% anethole in the diet) mated with control F_1 males (from the 4-generation study) [Le Bourhis, 1973b]. Litters born from treated females were exchanged with litters from control females at birth and reared by the new dams. No significant difference in body weights of pups from those nursed by mothers of the same group, regardless from which group they were born, was reported and final body weights of pups born from treated dams but raised by control dams regained normal values by day 28. The results indicated that postnatal growth is not directly affected by anethole exposure, but is a result of the nutritional status of the dams.

Given the comprehensiveness of this study and the concurrence with the results of the developmental study described in section 3.4.5, no further testing on the possible reproductive toxicity of anethole is recommended.

3.4.5 Teratogenicity/Developmental Toxicity

In a developmental and reproductive screening test [Argus Research Laboratories, 1992], groups of female rats were gavaged with anethole 0, 35, 175, or 350 mg/kg bw/day in corn oil for 7 days prior to co-habitation with male rats until day 4 of lactation. Similar to the 4-generation study reported in section 3.4.4, the only notable effects were reduced mean body weights and decreased feed consumption in high-dose rats. These effects were seen to some extent in rats gavaged with anethole 175 mg/kg bw/day, but only reached statistical significance in the early part of the study. At the high dose (350 mg/kg

bw/day), the number of liveborn pups was significantly decreased, the number of stillborn pups was significantly increased, the number of pups dying on day 1 and days 2-4 was significantly increased, the viability index (number of live pups on postpartum day 4/number of liveborn pups on postpartum day 1) was significantly decreased, the number of surviving pups/litter on postpartum day 4 was significantly decreased, the live litter size on postpartum day 4 was significantly decreased, and pup weight/litter on postpartum day 1 was significantly decreased compared to controls. No anomalies and no other effects were reported. The authors determined the maternal and developmental NOAEL to be 35 and 175 mg/kg bw/day, respectively, and the maternal and developmental lowest observable adverse effect level (LOAEL) to be 175 and 350 mg/kg bw/day, respectively. Anethole did not cause any effects on the rat fetus at doses below those causing maternal toxicity (reduced body weight and feed consumption).

No additional testing is recommended given the adequacy of this study.

3.4.6 New Testing Required

None.

3.5 TEST PLAN TABLE

Chemical	Physical-Chemical Properties				
	Melting Point	Boiling Point	Vapor Pressure	Partition Coefficient	Water Solubility
CAS No. 4180-23-8 *trans*-Anethole **CAS No. 104-46-1** Anethole (unspecified isomer)	A, Calc	A, Calc	A, Calc	Calc	A, Calc

Chemical	Environmental Fate and Pathways			
	Photodegradation	Stability in Water	Biodegradation	Fugacity
CAS No. 4180-23-8 *trans*-Anethole **CAS No. 104-46-1** Anethole (unspecified isomer)	Calc	NA	A	Calc

Chemical	Ecotoxicity		
	Acute Toxicity to Fish	Acute Toxicity to Aquatic Invertebrates	Acute Toxicity to Aquatic Plants
CAS No. 4180-23-8 *trans*-Anethole **CAS No. 104-46-1** Anethole (unspecified isomer)	A, Calc	A, Calc	A, Calc

Chemical	Human Health Data					
	Acute Toxicity	Genetic Toxicity *In Vitro*	Genetic Toxicity *In Vivo*	Repeat Dose Toxicity	Repro-ductive Toxicity	Develop-mental Toxicity
CAS No. 4180-23-8 *trans*-Anethole **CAS No. 104-46-1** Anethole (unspecified isomer)	A	A	A	A	A	A

Legend	
Symbol	**Description**
R	Endpoint requirement fulfilled using category approach, SAR
Test	Endpoint requirements to be fulfilled with testing
Calc	Endpoint requirement fulfilled based on calculated data
A	Endpoint requirement fulfilled with adequate existing data
NR	Not required per the OECD SIDS guidance
NA	Not applicable due to physical/chemical properties
O	Other

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